THE ANALYSIS OF URINE UBIQUITINATED PROTEINS REVEALED IMPAIRED ACTIVATION OF COMPLEMENT AND COAGULATION CASCADES IN DIABETIC NEPHROPATHY

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BACKGROUND AND AIMS

Diabetic nephropathy (DN) is a common microvascular complication occurring in approximately 20-40% of patients with type 2 diabetes mellitus (T2DM) [1]. It is characterized by the progressive impairment of glomerular function leading to end-stage renal failure with resulting need of dialysis or kidney transplantation. Hyperglycaemia, kidney inflammation [2] and oxidative stress [3] are crucial in promoting the development and progression of DN that, in turn, may lead to increased release of urinary ubiquitin and impaired ubiquitination of specific target proteins [4]. We aimed at demonstrating a significant correlation between the progression of renal damage in DN and the selective release of ubiquitinated proteins (ubi-prot) in the urine of affected patients in order to provide new urinary biomarkers of DN and to explore new pathogenetic mechanisms of the disease.

METHODS

Samples: Urine samples from 12 T2DM patients [3 normoalbuminuric (NORMO), 3 microalbuminuric (MICRO), 3 Kimmestiel-Wilson DN and 3 not DN chronic kidney Disease (CKD)], were collected at Section of Nephrology of the Dept. of Surgery and Medical Sciences of the University of Foggia . Each sample was concentrated and protein content was assessed spectrophotometrically.
Ubi-Prot Isolation: Urine Ubi-prot were purified from 500 μg total protein of each sample by dynabeads immunoprecipitation with a specific anti-ubiquitin antibody, then an aliquot of the samples was analysed by 1D-SDS page and Coomassie-stained to highlight ubi-prot excretion in each group.
Ubi-prot sequencing: the remaining aliquot of samples underwent trypsin digestion and MS analysis by LTQ Orbitrap XL™ Mass Spectrometer for protein sequencing.
Post-processing analysis: MaxQuant software was used for proteomic dataset processing.
Pathway analysis: String software was used for bioinformatics pathway analysis in order to identify function and pathway-correlated proteins.

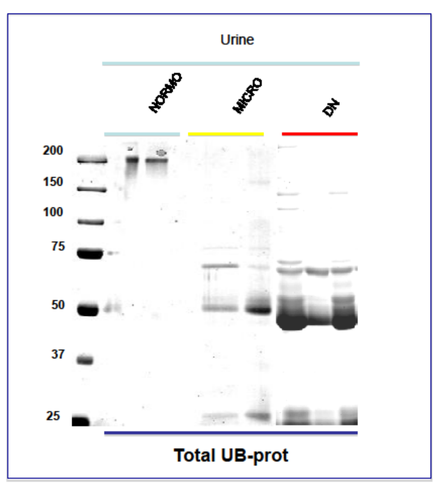

Figure 1 Increased excretion of ubi-prot in DN patients
Representative WB of ubi-prot excretion in NORMO, MICRO and DN patients after selective IP from urine samples.

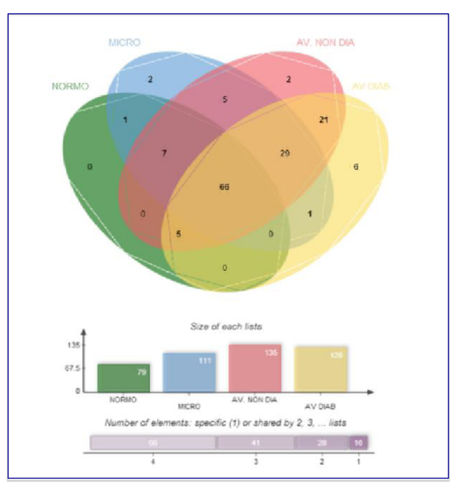

Figure 2 VENN diagram on identified ubi-prot
Ubi-prot shared by two or more groups are represented by the VENN diagram (upper). The total number of identified ubi-prot is reported in the histogram. Sixty-six proteins were common to all groups, 41 were common to 3 groups, 28 to 2 groups while 10 proteins were unique. Interestingly, 6 ubi-prot were excreted in only DN, 2 in non diabetic CKD (NON DIA) and 2 in MICRO group.

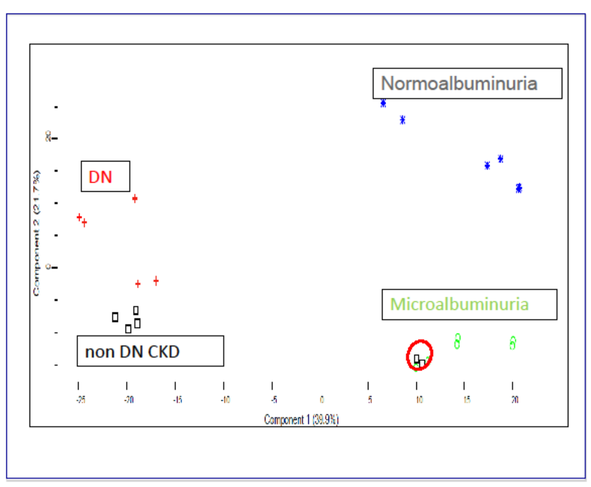

Figure 2 Principal Components Analysis based on urine ubi-prot.
The PCA analysis shows an appreciable segregation of the groups with the exception of one non DN CKD patient that had a ubi-prot profile, very similar to those of the microalbuminuric patients (highlighted by the circle)

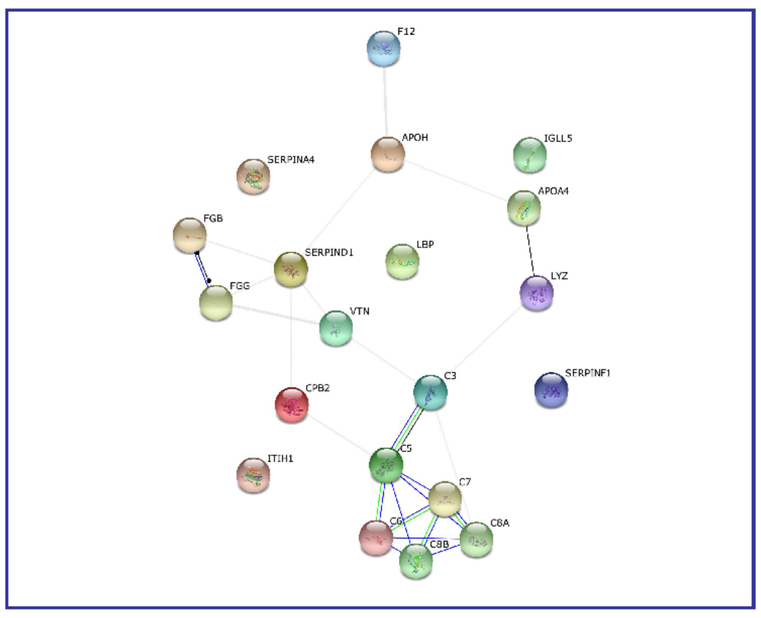

Figure 3 Network Display of the significant ubi-prot in DN.
The colors represent the type of action. Blue lines indicate the binding, the black ones mean reaction, while the green lines represent the activation

Term	NumberOfGenes	p-value	p-value_fdr	p-value_bonferroni
Complement activation, classical pathway	19	6.23E-38	7.58E-34	7.86E-34
Protein activation cascade	20	1.2E-37	7.58E-34	1.52E-33
Humoral immune response mediated by circulating immunoglobulin	19	3.74E-37	1.57E-33	4.72E-33
Immunoglobulin mediated immune response	19	1.28E-34	4.02E-31	1.61E-30
Complement activation	18	1.85E-34	4.26E-31	2.34E-30
B cell mediated immunity	19	2.03E-34	4.26E-31	2.56E-30
Lymphocyte mediated immunity	19	1.09E-32	1.96E-29	1.37E-28
Adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	19	3.6E-32	5.68E-29	4.54E-28
Leukocyte mediated immunity	19	3.21E-31	4.5E-28	4.05E-27
Adaptive immune response	19	1.18E-30	1.48E-27	1.48E-26
Humoral immune response	18	2.99E-29	3.42E-26	3.77E-25
Activation of immune response	19	2.63E-25	2.76E-22	3.31E-21
Immune effector process	19	1.82E-24	1.77E-21	2.3E-20
Positive regulation of immune response	18	1.45E-21	1.31E-18	1.83E-17
Regulation of immune response	19	4.26E-20	3.58E-17	5.37E-16
Positive regulation of immune system process	18	3.47E-19	2.73E-16	4.37E-15
Fc-gamma receptor signaling pathway involved in phagocytosis	12	4.84E-19	3.19E-16	6.1E-15
Fc receptor mediated stimulatory signaling pathway	12	4.84E-19	3.19E-16	6.1E-15
Immune response-regulating cell surface Receptor signaling Pathway involved in phagocytosis	12	4.84E-19	3.19E-16	6.1E-15
Innate immune response	19	5.1E-19	3.19E-16	6.43E-15
Fc-gamma receptor signaling pathway	12	5.31E-19	3.19E-16	6.7E-15
Phagocytosis	13	9.28E-19	5.32E-16	1.17E-14

Table 1 List of the significant functional pathways activated in Diabetic Nephropathy.

RESULTS

The progression of renal damage in T2DN correlates with increased excretion of urinary ubi-prot
Ubiquitome analysis revealed a remarkable increase of Ubi-prot in urine of DN patients (figure 1). Ubi-prot were almost absent in urine of NORMO patients and slightly visible in MICRO patients.

Mass Spectrometry (MS) analysis reveals distinct set of proteins correlated to each phases of the renal damage in T2DM
MS analysis allowed the overall identification of 79 ubi-prot in NORMO pts, 111 in MICRO pts, 128 in DN and 135 in not DN CKD (figure 2). Principal Component Analysis (PCA) allowed to clearly distinguish all the groups according to the rate of ubi-prot excretion (figure 3)

The activation of Complement classical pathway and coagulation are prominent processes in DN
Twenty-five ubi-prot were significantly more excreted in DN group compared to MICRO and not DN CKD. Most of them were components of the membrane attack complex (C5b, C6, C7 and C8) or were implied in the activation of coagulation processes (Vitronectin, Fibrinogen and Heparin cofactor II). Among functional pathways, the Complement was the most represented (table 1 and figure 3).

CONCLUSIONS

Our findings would suggest that the impaired activation of complement and coagulation cascades may take part into DN pathogenesis. A more detailed characterization of the meaning of the ubiquitination of these proteins may allow clarifying the role of such post-trasductional modification in the regulation of these pathways.

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